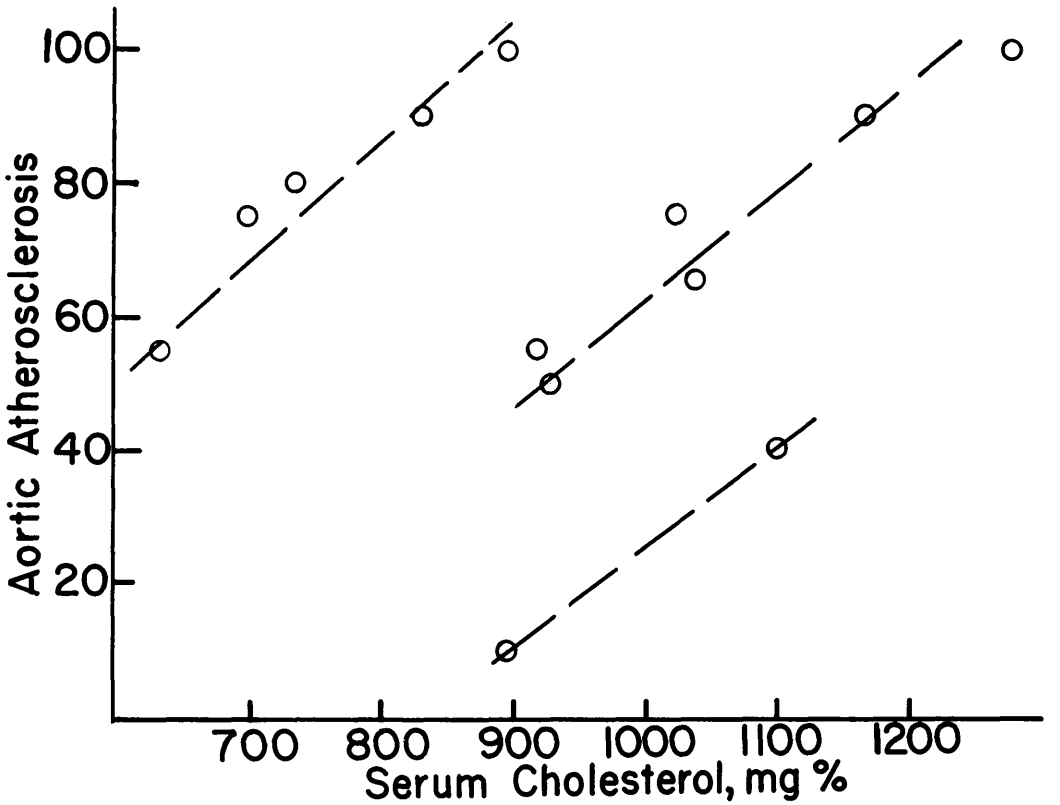

FIG. 1. Serum cholesterol level after 35 weeks on the diet *vs* degree of aortic atherosclerosis. The data suggest that the rabbits may represent 3 different populations of varying degrees of susceptibility.

1. Wertheimer, E., Shapiro, B., Physiol. Rev., 1948, v28, 451.

2. Jeanrenaud, B., Metabolism, 1961, v10, 535.

3. Havel, R. J., Proceedings of the First International Pharmacological Meeting, Horning, E. C., ed., Pergamon Press, Oxford, 1961, 43-65.

4. Correll, J. W., Science, 1963, v140, 387.

5. Fisher, C. M., Gore, I., Okabe, N., White, P. D., J. Neuropath. Exp. Neurol., 1965, v24, 455.

6. Carpenter, K. J., Gotsis, A., Hegsted, D. M., Clin. Chem., 1957, v3, 233.

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Interaction of the Lathyrogen Beta-Aminopropionitrile (BAPN) with a Copper-Containing Amine Oxidase.* (31763)

ROY C. PAGE AND EARL P. BENDITT

Department of Pathology, Medical School, University of Washington, Seattle

Lathyrogens appear to exert their effect on connective tissues by inhibiting the production of new covalent crosslinks in collagen and elastin(1,2,3). The excess soluble collagen which accumulates in the tissues of experimental animals after administration of lathyrogens seems to be intrinsically normal, but is deficient in aldehyde content(4,8). Substances which are thought to be the covalent interchain crosslinks of elastin and the intra-

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molecular crosslink of collagen have recently been isolated and partially characterized. These compounds derive from lysine, and their formation appears to follow oxidative deamination of lysyl side chains(5-8), presumably by a copper-containing amine oxidase(8). There is evidence suggesting that this deamination is impaired in the lathyritic animal(4,8), and it appears likely that lathyrogens may function by inhibiting the amine oxidase activity which is apparently necessary for crosslink formation.

In the absence of direct access to the enzyme presumably involved in crosslink formation and in order to test this hypothesis, we have examined the activity of pig plasma amine oxidase with respect to a lathyrogen. The plasma enzyme contains copper and possesses many other characteristics expected of the proposed connective tissue oxidase(9-13).

Methods. The method of Yamada and Yasunobu(13) was followed throughout the collection and preparation of plasma and precipitation of the enzyme with ammonium sulfate. Activity was assayed by the spectrophotometric method of Weissbach *et al*(14) using kynuramine as substrate. Incubation mixtures contained 0.5 ml of whole citrated plasma or ammonium sulfate-prepared enzyme, 0.15 mMole of sodium phosphate buffer pH 7.4, 0.3 μMole of kynuramine and water to 3.0 ml. All solutions were made up in ion-free water. Triplicate incubations were carried out in a circulating water bath at 37°C for one hour in glass-stoppered, 3 ml cuvettes. Control cuvettes contained all of the ingredients except substrate. The change in absorbence at 360 mμ was read in a spectrophotometer at 10-minute intervals and the amount of substrate used calculated, using the molar absorptivity of kynuramine of 3,520. Protein concentration was determined by the method of Waddell(15). One unit of enzyme activity was defined as the amount necessary to catalyze a change of 0.001 absorbence unit per minute under the assay conditions noted. The enzyme used in the experiments to be reported was purified 4- to 6-fold.

Inhibition studies were carried out under the conditions described above, except that incubation mixtures contained 1.9 units of enzyme, 3 to 300 μMoles of beta aminopropionitrile HCl (BAPN) and the amount of substrate was varied from 0.06 to 0.6 μMole. Solutions of kynuramine and BAPN HCl were adjusted to neutrality with NaOH prior to use. Three to four separate incubations were made for each concentration of substrate and inhibitor and the mean value used to calculate the slopes and intercepts of a reciprocal plot from which the kinetic constants were obtained.

Manometric assay of the enzyme was carried out in the Warburg apparatus at 37°C. Each flask contained 8.7 units of enzyme, 5 to 50 μMoles of BAPN HCl, 60 μMoles of potassium phosphate buffer pH 7.2 and water to 2.9 ml. In order to trap liberated ammonia, the center well contained 0.1 ml of 2% sodium borate pH 5.1 prepared according to Conway(16). Ammonia production was determined by microdiffusion and titration.

The protein of incubation mixtures from the manometric studies was precipitated by addition of an equal volume of 25% TCA and the mixtures clarified by centrifugation at 15,000 × *g* for 30 minutes. Spectra (Fig. 2) were obtained on portions of this supernatant. The supernatants were examined for cyanoacetaldehyde by the azobenzenephenylhydrazine sulfonic acid spot test(17) and tested for phenylhydrazone formation by the method of Blaschko *et al*(10). The incubation mixtures were tested for cyanoacetic acid content by the colorimetric method of Sievert *et al*(18).

A batch procedure was used to produce sufficient oxidation product for isolation. The contents of this incubation mixture were identical to those used in the manometric assay except that all ingredients were increased 10-fold and 400 μMoles of BAPN were used. The incubation mixture in a 125 ml flask was perfused for 10 minutes with oxygen and incubated overnight at 37°C in a metabolic shaker. The protein was then precipitated by addition of 6 volumes of ethanol and removed by centrifugation at 15,000 × *g* for 30 minutes. The supernatant was dried *in vacuo* and the residue taken up in methanol. Upon addition of 3 volumes of dry ether at

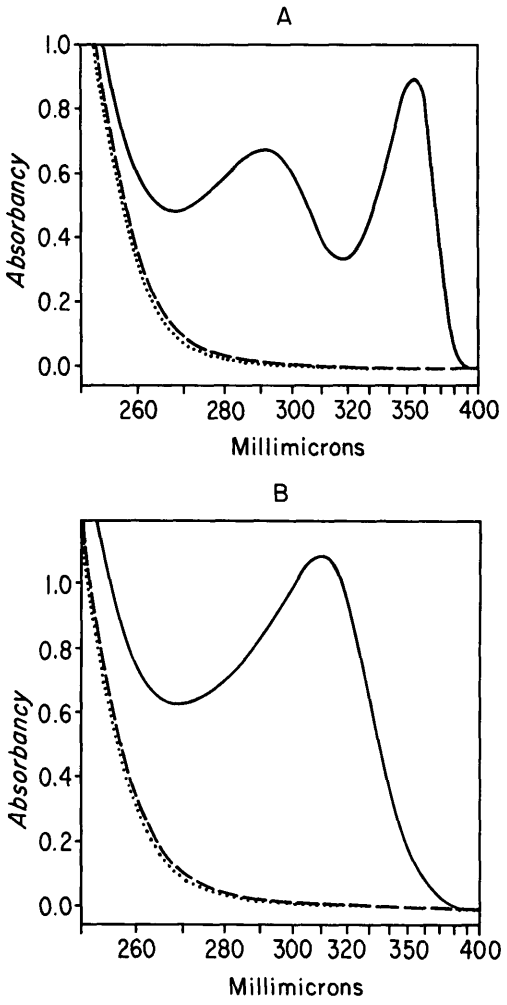


FIG. 1. A. Spectra of 12% TCA supernatants of manometric incubation mixtures; B. same solutions adjusted to pH 8.0 with NaOH; (——) enzyme and BAPN, (....) BAPN with no enzyme (- - - -) enzyme with BAPN added just prior to protein precipitation.

0°C a yellow flocculent precipitate formed and was removed by centrifugation. This material was reprecipitated twice from methanol and dissolved in dry 3% methanolic HCl to yield a clear yellow, fluorescent solution with a single absorption maximum at 370 mμ. Fluorometric studies were carried out on this solution. The material was then precipitated with ether and dried over calcium chloride. About 15 mg of dry material was obtained. Thin layer chromatography of the product was carried out on silica gel H using butanol: acetic acid:water (12:3:4) as solvent and stained with ninhydrin. Elemental analysis of the material was done by the Schwarzkopf Microanalytical Laboratory, N. Y.

Results. The effect of various concentrations of BAPN HCl on the oxidative deamination of kynuramine by pig plasma amine oxidase is illustrated in Table I. Concentrations of BAPN HCl up to 5 mM progressively increased Km but did not significantly alter Vmax, while higher concentrations altered both Km and Vmax. Therefore, concentrations of lathyrogen below 10 mM appear to inhibit enzyme activity competitively, while higher concentrations inhibit by some other mechanism. A concentration of 100 mM BAPN HCl completely inhibited enzyme activity.

A series of dialysis experiments was done to determine if BAPN is irreversibly bound to the enzyme. Enzyme with kynuramine substrate was incubated for one hour in the presence of 100 mM BAPN HCl, or in controls with an ionic equivalent of BAPN in the form of phosphate buffer, then dialyzed overnight and again assayed. Control and BAPN-incubated enzyme showed essentially the same activity after dialysis, suggesting that the lathyrogen, even at this high concentration, is not irreversibly bound to the enzyme.

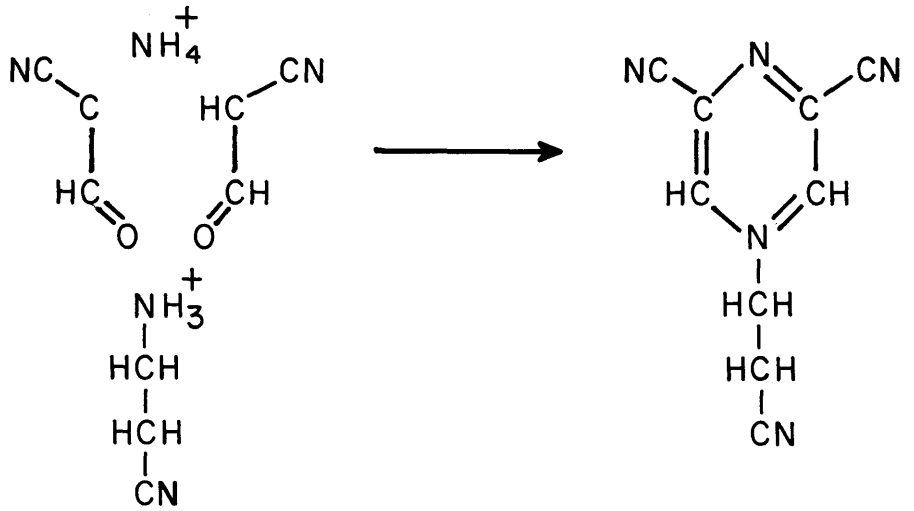
BAPN possesses an amino group which one might expect to be suspectible to oxidative removal by amine oxidase. The compound was tested for substrate activity by following oxygen consumption and ammonia production in the Warburg apparatus. Oxygen was used and ammonia produced; therefore, BAPN is not only a competitive reversible inhibitor, it forms a productive complex with the active site of this amine oxidase.

TABLE I. Effect of Various Concentrations of Lathyrogen on Kinetic Constants for Oxidative Deamination of Kynuramine by Pig Plasma Amine Oxidase.

mM lathyrogen concentration	Vmax, μMoles/min	Km, mM
0	.70	31.3
1	.70	37.0
5	.74	92.5*
10	.59*	101.6*
100	—	—

* $P < .01$.

Possible Product of Enzymatic Oxidative Deamination of BAPN



Ammonia
BAPN
Cyanoacetaldehyde

2,6-dicyano-4-cyanoethyl pyrazine

FIG. 2.

BAPN is converted *in vivo* to cyanoacetic acid, presumably by oxidative deamination and subsequent oxidation by aldehyde oxidase(19). In spite of intensive efforts we were not able to demonstrate either cyanoacetic acid or cyanoacetaldehyde in the incubation mixtures using BAPN as substrate. In addition, BAPN was not consumed quantitatively regardless of the incubation time, and ammonia production was always about 15 to 25% less than expected from the oxygen consumption.

The spectra of supernatants of the incubation mixtures containing BAPN as substrates and of appropriate controls, shown in Fig. 1, demonstrate the presence of a UV-absorbing oxidation product. We have isolated and partially characterized this product, and its properties are shown in Table II. This unstable material when placed in solution in 3% methanolic HCl absorbs maximally at 370 mμ and emits at 475 mμ. It migrates on thin layer silica gel as multiple ninhydrin positive spots. Chromatograms of solutions of the material in which breakdown has been allowed to occur show the regeneration of a spot with the migratory and staining characteristics of authentic BAPN.

Discussion. The amine oxidase of pig plasma was selected as a model of the proposed

TABLE II. Properties of the Product of Oxidative Deamination of BAPN by Amine Oxidase.

1. Bright yellow color in visible light and fluorescent under UV light.
2. Unstable and apparently light-sensitive.
3. Absorption spectra:
   (a) NaOH pH 8.0: maximum at 310 mμ
   (b) TCA 12%: maxima at 290 and 355 mμ
   (c) Ethanol: maxima at 278 and 320 mμ
   (d) Ethanolic HCl: maximum at 370 mμ
4. Emission spectra: absorbs at 370 mμ and emits at 475 mμ.
5. Nitrogen to carbon ratio of 0.64; no discrete melting point.
6. Migrates on silica gel in both acidic and basic solvents and stains red with ninhydrin.
7. Ammonia and unoxidized BAPN appear to be bound in the complex.

connective tissue amine oxidase. This appears to be a logical choice since an enzyme of this type has wide species distribution(10); it will deaminate methyl-6-aminohexanoate, a substrate resembling lysyl residues (unpublished observations); and it functions optimally near neutral pH and in an ionic and protein environment comparable to that encountered in the connective tissues(9,11). The enzyme contains copper(10,11,13), and, in the copper-deficient pig, which exhibits defects in crosslink formation in elastin, its activity is dramatically decreased(12).

The proposed connective tissue amine oxidase likely functions in the connective tissue space in the presence of extraneous protein largely derived from plasma(17). Consequently, the enzyme used was purified only 4- to 6-fold since all we desired was increased activity for ease of measurement.

The data presented show that BAPN inhibits the activity of the enzyme competitively and reversibly and forms a productive complex with the active site of the enzyme. The apparent affinity of the enzyme for BAPN is in the direction and order of magnitude expected of a competitive enzyme inhibitor. The large size and possible particulate nature of collagen and elastin provide factors which would reduce the collision frequency of the enzyme with its substrate and favor the action of small-size compounds such as BAPN as a competitor and inhibitor of oxidative deamination.

The fact that neither cyanoacetic acid nor cyanoacetaldehyde could be detected in the incubation mixtures using BAPN as substrate either with the highly sensitive spot test used or by phenylhydrazone formation is at first puzzling. The further findings that a significant portion of the ammonia produced by the reaction cannot be recovered and that a product can be isolated which is unstable and upon breakdown gives rise to a material with the chromatographic and staining properties of authentic BAPN are compatible with the formation *in vitro* of a condensation product containing cyanoacetaldehyde, ammonia and BAPN. There are several possible structures for such a product. However, the data presented above, along with the UV-absorption and fluorescence spectra, the instability, and nitrogen to carbon ratio, lead us to propose a structure of the type presented in Fig. 2. This compound is one of several possible compounds and is shown only to illustrate the type of structure we believe may be present.

The properties of the compound we have isolated, and the fact that *in vivo* BAPN is converted to cyanoacetic acid, suggest the existence of a highly reactive aldehyde intermediate in BAPN metabolism which is capable of undergoing condensations with amines. This provides another possible way in which BAPN could prevent collagen or elastin crosslinking. Cyanoacetaldehyde could undergo Schiff base formation with functional lysyl amino groups of collagen and elastin molecules and block further enzyme activity, or BAPN may undergo the same type of reaction with previously produced aldehydes which have not yet undergone condensation. Although it has been reported that when labeled BAPN is administered to experimental animals significant amounts of radioactivity are not recovered in the extracted, purified collagen(20), more recent data(21) indicate that Schiff bases of the type proposed are unstable in some cases under the conditions used in the isolation and purification of collagen in these experiments.

Summary. Pig plasma amine oxidase, an enzyme thought to closely resemble the oxidase functional in collagen and elastin crosslinking, was assayed in the presence of a lathyrogen. BAPN was shown to inhibit enzyme activity competitively and reversibly and to form a complex with the active site of the enzyme. The product of the enzymatic oxidative deamination of BAPN was isolated and partially characterized. Its properties indicate the existence of a highly reactive aldehyde intermediate in BAPN metabolism. The data presented suggest two likely alternatives for the mechanism of action of BAPN. (a) BAPN may compete with native substrate molecules for the active site of the amine oxidase functional in crosslinking, or (b) BAPN or a highly reactive aldehyde derived from BAPN by the action of amine oxidase may react with the functional groups of strategic lysyl side chains of collagen and elatstin and

block enzyme activity and crosslink formation.

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1. Smiley, J. D., Yeager, H., Ziff, M., J. Exp. Med., 1959, v116, 45.

2. Page, R. C., Benditt, E. P., Lab. Invest., 1966, v15, 1643.

3. Bhatnagar, R. S., Doctoral Dissertation, Duke Univ., 1964.

4. Page, R. C., Benditt, E. P., Proc. Soc. Exp. Biol. and Med., 1967, v124, 459.

5. Partridge, S. M., Elsden, D. F., Thomas, J., Dorfman, A., Telser, A., Pei-Lee Ho, Biochem. J., 1964, v93, 30c.

6. Franzblau, C., Sinex, F. M., Faris, B., Lampidis, R., Biochem. Biophys. Res. Commun., 1965, v21, 575.

7. Miller, E. J., Martin, G. R., Piez, K. A., ibid., 1964, v17, 248.

8. Bornstein, P., Kang, A. H., Piez, K. A., Proc. Nat. Acad. Sci., 1966, v55, 417.

9. Blaschko, H., Enzymes, 1963, v8, 337.

10. Blaschko, H., Friedman, P. J., Hawes, R., Nilsson, K., J. Physiol., 1959, v145, 384.

11. Buffoni, F., Blaschko, H., Proc. Roy. Soc. (London), 1964, 151-B, 153.

12. Blaschko, H., Buffoni, F., Weissman, N., Carnes, W. H., Coulson, W. F., Biochem. J., 1965, v96, 4c.

13. Yamada, H., Yasunobu, K. T., J. Biol. Chem., 1962, v237, 1511.

14. Weissbach, H., Smith, T. E., Daly, J. W., Witkop, B., Udenfriend, S., ibid., 1960, v235, 1160.

15. Waddell, W. J., J. Lab. Clin. Med., 1965, v48, 311.

16. Conway, W., Microdiffusion Analysis and Volumetric Error, Crosby, Lockwood & Son, Ltd., London, 1957.

17. Feigl, F., Spot Tests in Organic Analysis, Elsevier Publishing Co., N. Y., 1956, p213.

18. Sievert, H. W., Lipton, S. H., Strong, F. M., Arch. Biochem. & Biophys., 1960, v86, 311.

19. Lalich, J. J., Science, 1958, v128, 206.

20. Orloff, S. D., Gross, J., J. Exp. Med., 1963, v117, 1009.

21. Homquist, W. R., Schroeder, W. A., Biochemistry, 1966, v5, 2489.

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A Molecular Defect in Lathyritic Collagen.* (31764)

ROY C. PAGE AND EARL P. BENDITT

Department of Pathology, Medical School, University of Washington, Seattle

Experimental lathyrism is a disease of connective tissues, characterized by the occurrence of hernia, aneurysm, and skeletal deformity(1). Underlying the disease is a dramatic increase in the amount of soluble collagen present in the tissues, apparently resulting from a decreased degree of covalent, interchain crosslinking(2,3). A similar defect appears to occur in elastin.

One of the basic problems concerning this disease has been the question of whether lathyrogens inhibit new crosslink formation or cause a rupture of previously-formed crosslinks. The answer to this question is important since it will determine how and where one looks for the molecular defect. Tracer kinetic analysis has been used but the results have led to conflicting conclusions(4,5). This problem was partially resolved by determining the relative rates of accumulation of soluble, insoluble, and total collagen in whole normal and lathyritic chick embryos(6). The data showed that the most likely source of lathyritic collagen is newly aggregated fibrils synthesized after lathyrogen administration. Therefore, lathyrogens seem to exert their effect on connective tissues by causing inhibition of new covalent crosslink formation rather than by rupturing previously-formed crosslinks.

There appear to be two likely mechanisms by which lathyrogens may inhibit the formation of new covalent crosslinks. (a) Lathyrogens may induce structural defects in the crosslinking portion of collagen and elastin molecules, or (b) they may affect a compo-

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